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STUDY ON THE CLASSIFICATION OF STREPTOCOCCI

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The purpose of this study is the classification of streptococci by the appearance of deep colonies in blood agar plates of uniform composition and thickness; and a further classification by the fermentation reactions in inulin, lactose, mannite, raffinose, saccharose and salicin; and last, by a study of the agglutination reactions in serum of immunized animals. Lastly an attempt is made to show the relationship of the serologic groups to the blood agar and sugar reaction groups. Some additional properties have been investigated and the general results noted in their bearing on the main issues.

Brown [1] classifies the streptococci, by the growth and action of deep colonies in blood agar, into three main groups: alpha, beta and gamma and a subgroup, alpha prime.

Alpha colonies are those which in other classifications are called "viridans" or "green" and which in deep colonies produce a 0.8-1.0 mm. double-convex, oval, or complex colony, surrounded by a small zone of green colored red cells at the end of 48 hours' incubation. The complex colony is not definitely double-convex or oval, but a seeming combination of two such colonies. It appears at first as if there are two colonies lying at right angles to each other, one superimposed on the other. When placed in the icebox for from 24-48 hours, there appears surrounding the area of green coloration a zone of hemolysis, wherein no red cells or color is visible. Further incubation produces a second ring of green formation and a secondary zone of hemolysis occurs if it is placed again in the icebox. There are, then, alternating concentric zones of hemolysis with the first zone of green immediately surrounding the colony. The number of zones possible seems in the main limited to the number of incubations and refrigerations, although the drying of the medium and the lack of available food supply serves as a check on the growth. This description in the main fits all the alpha streptococci. Many of them, however, are apparently limited to smaller

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[1] Monograph 9, Rockefeller Institute, 1919.

colonies and proportionately smaller zones, and some do not elaborate as clear-cut zones as others. Most of these differences, however, seem to be one of degree and not of kind, so that a further differentiation would lead to confusion rather than clarity.

Brown, however, does make one further differentiation—alpha prime—in the alpha group. This he describes as a colony in which the zone of hemolysis is not clear-cut macroscopically. This is accounted for, when examined microscopically, by finding in the hemolytic zones scattered green-colored red cells which are the survivors of the hemolytic action which destroyed the larger number of the cells. Next to the colony the number of cells is slightly greater than in the more distant portions of the agar. We have never seen any colonies to fit this description.

In the beta group fall those streptococci which in other classifications have been described as "hemolytic." The deep colonies, in size and shape resembling the alpha type, surround themselves on the plate with a zone of clear agar containing no visible cells or hemoglobin. This zone varies in size from 3-8 mm. at the end of 48 hours' incubation. Further incubation or cultivation in the icebox produces no change in the type of hèmolysis. The diameter of the zone of hemolysis increases in most cases on further incubation of body or refrigerator temperature.

Those streptococci which produce no change in the blood agar medium and which are known in the older groupings as "nonhemolytic" constitute the gamma group.

Since the terms alpha and beta are nondescriptive and the general tendency has been recently to use the more descriptive terms "green" and "hemolytic," we shall hereafter refer to the groups by the latter terms.

The numerical classification used in this study for the grouping of the sugar reactions is Brown's,[1] as shown in table 1.

About 90 organisms were isolated. The sources of the cultures are given in table 2. They were personally collected, with the exception of those which came from the laboratory of the University Hospital. All of the cultures were fished from surface platings on blood agar and isolated as hemolytic streptococci; and some were classified on the basis of their reaction in sugar mediums, as suggested by Holman,[2] using Hiss serum water medium with 1% of inulin, lactose, mannite and salicin.

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[2] Jour. Med. Res., 1916, 34, p. 315.

Mediums.—The agar medium used in this study was composed of 2 gm. of Liebig's extract of beef, 10 gm. of Witte's peptone and 15 gm. of Difco agar-standardized to the liter. This medium was made by dissolving the agar in boiling water and adding the other ingredients, while the medium was cooling to 60 C., securing thorough solution by rapid stirring. When cool enough, one beaten egg per liter was added and the whole again brought to the boiling point, filtered and the reaction adjusted to P_H 7.4. When sterilized at 15 lbs. for 15 minutes, the final reaction which was tested on several occasions never fell below P_H 7. The medium was tubed—12 c c to the tube—or placed in small flasks — 75 c c to the flask — and stored in the icebox until used. Evaporation and shrinkage were slight.

TABLE 1

TABULATION OF STREPTOCOCCI (AFTER BROWN[1])

Mannite	Lactose	Salicin	Groups	Type in Blood Agar Green, hemolytic or nonhemolytic							
—	+	+	1	S. mitis S. pyogenes			Green Hemolytic				
		—	2	S. salivarius S. anginosus			Green Hemolytic				
	—	+	3	S. equinus S. equi			Green Hemolytic				
		—	4	S. ignavus S. subacidus			Green Hemolytic				
+	+	+	5	S. faecalis S. infrequens			Green Hemolytic				
		—	6								
	—	+	7	S. alactosus			Hemolytic				
		—	8								
				.1	.2	.3	.4	.5	.6	.7	.8
Raffinose				—	+	—	+	—	+	—	+
Inulin				—		+		—		+	
Saccharose				+				—			

The blood was secured in a sterile Erlenmeyer flask—250 c c—fitted as an aspirating bottle. The needle was capped with a glass tube before sterilization in the autoclave. The blood was taken from the jugular vein of the sheep. It was then defibrinated in the bottle and pipetted to sterile test tubes, where, if no contamination occurred, it gave satisfactory results for a period of at least one week.

The blood was added to the agar, 0.6 c c to a 12 c c tube, just before pouring the plates; 4 c c to a 75 c c flask of agar. This flask agar was poured into sterile tubes and used for 'stock cultures and other work requiring blood agar.

An attempt was made to secure the blood from the same sheep at each bleeding, but this was not always possible. On several occasions an animal was used, whose blood at each bleeding was grossly contaminated with green streptococci of the 1.1, 1.2 and 2.2 varieties. This contamination was observed on one occasion when the technic of securing the blood was the same as usual. The contamination, however, was laid to faulty technic. A second contamination occurred using the same sheep. Blood agar slants made from this bleeding were so grossly contaminated as apparently to preclude any other source than that of the blood. A special bleeding was now carried out and the same type of organisms isolated. No attempt was made to identify them again individually, but they were distinctly green streptococci with an extremely long chain formation in broth.

This particular occurrence is stressed because of the oft-repeated statements that the green and hemolytic organisms are capable of two appearances or rather of mutation. It seems possible that a green contamination in a tube might be the source of a so-called mutation. Green organisms, furthermore, develop but slowly and blood agar mediums should be incubated at least 72 hours—48 hours in the incubator and 24 hours at room temperature—before it is accepted for use.

Sugar Mediums.—At the beginning of this work, Hiss serum water medium was used and made on the standard formula: 1 part serum, 3 parts water, 1% sugar and litmus to color a satisfactory blue. This was found difficult to sterilize and in some of the sugars, particularly mannite, an odd shade of purple was observed that was confusing. Some of the organisms, however, were satisfactorily identified on this medium.

Trypsinized peptone water was then tried. This is a simple peptone water, treated in the incubator for three hours with trypsin—1 gm. to the liter of broth. At the end of this time it was brought to the boiling point and 2 gm. of meat extract added and the product filtered. The reaction is adjusted to P_H 7.2, 1% sugar and Andrade's indicator added. It was then sterilized at 15 pounds of pressure for 15 minutes. This medium when used with the cultures identified on the serum water medium produced the same results.

This medium had the advantage of being easy to prepare as the securing of suitable serum in large amounts was difficult; it was easier to sterilize, and the Andrade indicator gave a more delicate indication of the amount of acid production. The serum water medium coagulated in the presence of much acid, but a comparison of results on both mediums would indicate that a pronounced shade of red in the medium with the Andrade indicator is the equivalent of coagulation in the serum water medium. It also possessed the great advantage of showing growth by turbidity or sediment formation in tubes showing no acid formation.

Broth, plain, trypsinized, was made in the same way as the carbohydrate mediums, eliminating the fermentable substances. This, with the exception of one culture, produced a suitable growth for staining and sugar tube inoculation.

Broth, hormone, was prepared as follows: To 3 liters of water warmed to 60 C., were added 3 beaten eggs and 5 pounds of ground beef heart. To this warm mixture were added 2 liters of water containing the following dissolved materials: 50 gm. of peptone, 12.5 gm. of salt, 12.5 gm. of trypsinized peptone and 50 gm. of gelatin. The entire mixture was gradually raised to the boiling point without stirring after the beef has started to coagulate. It was boiled from 15-20 minutes. A small portion was filtered through filter paper and to the measured filtrate was added enough 10% HCl to cause the maximum precipitation.. HCl was added to the unfiltered portion of the mixture in the same proportion. It was boiled for a few minutes. The precipitation with HCl was repeated until no further precipitation occurred. When there was no further precipitate, the liquid was drawn from the coagulum and sterilized at 12 pounds of pressure for 15 minutes.

The next day the supernatant fluid was siphoned from the sediment. The reaction was adjusted to P_H 7.8-8 in the cold; 0.25% dextrose was dissolved in the mixture by boiling. It was poured into tall jars and allowed to settle until the supernatant fluid was perfectly clear, when it was tubed and sterilized. We sterilized in the autoclave and had apparently no disastrous results. This medium allows streptococci to grow luxuriantly.

Milk.—Certified milk was suitably colored with litmus, tubed and sterilized by raising the pressure to 15 pounds slowly, maintaining for 15 minutes and then allowing the autoclave to return to normal pressure by simply turning off the heat.

All of the mediums requiring peptone and meat extract were made from a 1 lb. jar of Liebig's extract and a 1 kg. bottle of Witte's peptone, both of which were from prewar stock.

Method of Study.—The cultures were studied as follows: A loopful of stock culture was inoculated into a series of 4 tubes by the usual serial tube method. The first 2 tubes of the series contained salt solution, the last 2, liquid blood agar, as many platings have shown the most successful plates to be those from the third and fourth dilutions. The plates were then incubated for 48 hours when the kind of hemolysis was recorded. If the plates all showed the same general kind of hemolysis and colony, two deep colonies were fished to broth by cutting out the surrounding agar in a small block; this block was then macerated on the wall of a broth tube and washed into the fluid portion of the broth. This method is mentioned because in a great many cases the ordinary stabbing of a deep colony seems insufficient to produce growth. The broth tubes were then incubated for 24 hours when the macroscopic appearance was noted and the stained preparations examined. A stock culture was made on blood agar and plants to sugar tubes by adding 0.5 c c of the broth culture to each sugar tube. Fermentations were noted at the end of 1, 3 and 7 days. Streptococci reach their full powers of fermentation at the end of 3 days if the sugar tubes receive a moderately heavy inoculation to overcome the initial lag. If the two fishings agreed in their sugar reactions, one stock culture was discarded.

If on the original plate there was present more than one kind of hemolysis or if the appearance of the two colonies varied materially in size or shape, two fishings of each general type were made and the procedure as given followed.

If the two sugar fermentations from the same type of colony did not agree, the two blood agar stock cultures were checked from the beginning by replating.

When the sugar reactions had been determined and agreed, one stock culture was again replated and put through the same procedure as a check.

The following standard was adopted: That on the original agar plate they agree in hemolysis; that the two fishings agree in sugar reactions; that one of the fishings when replated produce the same type of hemolysis; that this replating produce the same sugar fermentations from the two fishings as the original fishing. Later on, this was modified so that a culture yielding two colonies, alike on blood agar and in sugar reactions, was considered as determined.

Preparation of Antigens.—The first antigens used were blood-agar cultures suspended in salt solution. These, as a rule, were not smooth and tended to spontaneous clumping. Varying results were obtained with them. On occasions they were apparently agglutinated and again the same lot of antiserum produced no effect. Heat killed antigens were more satisfactory than live antigens.

Broth cultures were then tried. The majority of these, when the growth was heavy, were granular and unsatisfactory for that reason.

Hormone broth caused a heavy growth, which, if centrifuged and washed in Locke's solution (0.01% $CaCl_2$, 0.02% KCl in salt solution) within 18 hours after inoculation, produced a satisfactory product. The centrifuged and washed antigen was diluted with Locke's solution to a strength of 2 billion per c c and preserved with phenol to a strength of 0.5%.

Preparation of Antiserums.—In the preparation of antiserums, various methods were used, no one of which produced a very high titer in all of the animals used. In the first method we injected subcutaneously killed, blood agar cultures suspended in salt solution, followed by live cultures. Three doses were given on 3 successive days followed by an interval of 3 more days before resuming the injections. In most cases the animals developed open abscesses at the site of inoculation when large doses were given. This method produced low titers. These serums were titrated against blood agar antigens and the low titers may have been caused by unsatisfactory antigens.

Intravenous injections were then tried with antigens grown both in blood agar and broth. The most satisfactory results were obtained by using serum broth cultures in increasing doses every third day, using killed cultures for the first 3 injections. It was noticed on 3 occasions that after the inoculation of killed organisms, the first injection of a live broth culture of hemolytic organism caused the death of the rabbit no matter how small the dose. When these same organisms were centrifuged and resuspended in salt solution, there was no noticeable effect on the rabbits while some immunity was obtained. This disagrees with Kinsella[3] who found that washed streptococci produced no immunity.

The green organisms produced a much higher immunity than did the hemolytic type in the same number of injections. Some hemolytic cultures seem incapable of producing agglutinins. Several of the hemolytic type produced no measurable agglutinins after as many as 15 injections. The one hemolytic culture which has produced agglutinins had a titer of only 400, whereas titers as high as 3,000 were obtained in the green group. This seems to point to some fundamental difference between the two groups.

Chickens were used for the production of immune serums. They were immunized by our earlier methods and the titers were about the same as in the rabbit serums. It was thought possible that the chickens would be capable of producing high titers because of their protective substances against pneumococci.

Serologic Tests.—In making the agglutinations, 3 dilutions of each immune serum were used. The lowest dilution in each case was 1:50, the highest was one-half the titer of the serum with its homologous antigen and the third was a dilution halfway between the low and the high. Each agglutination was controlled by a plain antigen suspension in Locke's solution for the occurrence of spontaneous agglutination and by a normal rabbit serum in dilution of 1:50. The tubes were incubated in a water bath at 56 C. for 3 hours when readings were made. Agglutinations were read as positive if the controls showed no agglutination.

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[3] Jour. Exper. Med., 1918, 28, p. 151.

TABLE 2

CULTURAL AND AGGLUTINATIVE CHARACTERISTICS OF STREPTOCOCCI *

Classification Brown		Serial Number	Source	Milk			Agglutination Serums					Remarks
Green				Reaction	Coagulation	Reduction	14	19	22	M4	M9	
S. mitis	1.1	22	Tuberculosis sputum........	A	P	+	100	—	800	—	—	Nonpathogenic
	1.1	31	Influenza....................	A	+	+	100	—	400	—	—	
	1.1	35	Influenza....................	A	+	+	100	—	100	—	—	
	1.1	37	Influenza....................	A	+	+	400	—	400	—	—	
	1.1	43	Tuberculosis sputum........	A	+	+	400	—	400	—	—	
	1.1	48	Tuberculosis sputum........	A	+	+	50	—	—	—	—	
	1.1	128	Normal throat..............	A	+	+	400	—	400	—	—	
	1.1	UN/C	Contaminated blood agar...	A	+	+	100	—	100	—	—	
....	1.2	14	Peritonsillar abscess.........	A	+	+	1600	—	—	—	—	Nonpathogenic
	1.2	51	Tuberculosis sputum........	A	+	+	400	—	100	—	—	
	1.2	78	Empyema..................	A	P	—	—	—	—	—	—	Described by Smith and Brown, Hartzell and Henrici, Kligler, Broadhurst. Suggested name, S. secundomitis
	1.2	Fec 1	Sheep feces..................	A	+	+	—	—	—	—	—	
	1.2	Fec 4	Sheep feces..................	A	+	+	—	—	—	—	—	
	1.2	UN/B	Contaminated blood agar...	A	+	+	100	—	—	—	—	
....	1.3	52	Tuberculosis sputum.......	—	—	—	—	—	—	—	—	
	1.3	61	Normal throat..............	—	—	—	—	—	—	—	—	Acid production slight in sugars. Described by Smith and Brown, Broadhurst. Suggested name, S. tertiomoitis
	1.3	SH/D	Slaughter house blood.......	—	—	—	—	—	—	—	—	
....	1.4	23	Tuberculosis sputum........	A	—	—	—	—	—	—	—	Nonpathogenic
	1.4	65	Contaminated blood agar...	A	—	—	—	—	—	—	—	
	1.4	88	Influenza pneumonia........	a	—	—	—	—	—	—	—	Described by Kligler, Broadhurst. Suggested name, S. quartomitis. Acid production slight in sugars
	1.4	168	Tuberculosis sputum........	A	—	—	—	—	—	—	—	
	1.4	SH/B	Slaughter house blood.......	—	—	—	—	—	—	—	—	
....	2.2	100	Tuberculosis sputum........	A	—	—	—	—	—	—	—	Nonpathogenic
	2.2	101	Tuberculosis sputum........	A	—	—	—	—	—	—	—	
	2.2	UN/A	Contaminated blood agar...	A	+	+	100	—	—	—	—	Described by Smith and Brown, Hartzell and Henrici, Kligler, Broadhurst. Suggested name, S. secundosalivarius
S. equinus	3.1	62	Tuberculosis sputum........	—	—	—	—	—	—	—	—	
S. faecalis	5.1	35a	Contaminated blood agar...	—	—	—	—	—	—	—	—	Nonpathogenic
	5.1	54	Contaminated blood agar...	a	—	—	—	—	—	—	—	Acid production slight in sugars
	5.1	SH/A	Slaughter house blood.......	—	—	—	—	—	—	—	—	

....	5.3	67	Contaminated blood agar...	—	—	—	—	—	—	—	—	Acid production slight in sugars Described by Broadhurst Suggested name, S. tertiofaecalis
	5.3	68	Like 67.......................	a	—	—	—	—	—	—	—	
	5.3	70	Like 67.......................	—	—	—	—	—	—	—	—	
....	5.4	50	Tuberculosis sputum........	A	+	+	—	—	—	—	—	Described by Broadhurst. Suggested name, S. quartofaecalis. Diplococcus; no chains; no capsule; not bile-soluble
Hemolytic S. pyogenes	1.1	11	Tuberculosis sputum........	A	—	—	—	—	—	—	—	Pathogenic
	1.1	15	Boil..........................	A	—	—	—	—	—	—	—	
	1.1	16	Impetigo....................	A	—	—	—	—	—	—	—	
	1.1	17	Impetigo....................	A	—	—	—	—	—	—	—	
	1.1	19	Impetigo....................	A	—	—	—	300	—	—	400	Pathogenic
	1.1	20	Impetigo....................	A	—	—	—	—	—	—	—	
	1.1	21	Impetigo....................	A	—	—	—	—	—	—	—	
	1.1	26	Maternity case..............	a	—	—	—	—	—	—	—	
	1.1	27	Septicemia..................	a	—	—	—	—	—	—	—	
	1.1	28	Tuberculosis sputum........	A	—	—	—	—	—	—	—	
	1.1	57	Arm infection after vein removal.....................	A	—	—	—	—	—	—	—	
	1.1	79	Maternity case..............	A	—	—	—	—	—	—	—	
	1.1	114	Tuberculosis sputum........	A	—	—	—	—	—	800	—	
	1.1	117	Tuberculosis sputum........	A	—	—	—	—	—	—	—	
	1.1	150	Bott's disease abscess.......	A	—	—	—	—	—	—	—	
	1.1	172	Tuberculosis sputum........	A	+	+	—	—	—	—	—	
	1.1	174	Empyema..................	A	—	—	—	—	—	800	—	
	1.1	177	Impetigo....................	A	—	—	—	—	—	—	—	
	1.1	178	Impetigo....................	A	—	—	—	—	—	—	—	
	1.1	184	Impetigo....................	A	—	—	—	—	—	—	—	
	1.1	P	Abscess of axilla............	A	—	—	—	—	—	—	—	
	1.1	X	Cellulitis of hand...........	A	—	—	—	—	—	—	—	
....	1.5	173	Empyema..................	A	—	—	—	—	—	—	—	
	1.5	M12	Pasteurized 'A' milk.........	A	+	+	—	—	—	—	—	S. lacticus (Salter)
	1.5	M15	Pasteurized 'A' milk.........	A	+	+	—	—	—	—	—	S. lacticus (Salter)
S. anginosus	2.1	58	Tuberculosis sputum........	a	—	—	—	—	—	—	—	Pathogenic
S. subacidus	4.1	123	Medical ward...............	—	—	—	—	—	—	—	—	
S. infrequens	5.1	24	Septicemia..................	a	—	—	—	—	—	—	—	
	5.1	151	Pericardial fluid, pneumonia	a	—	—	—	—	—	—	—	
	5.1	175	Empyema..................	a	—	—	—	—	—	—	400	
....	5.5	M11	Pasteurized 'A' milk.........	A	+	+	—	—	—	—	—	Suggested name, S. quilacticus

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* The readings of the milk reactions were made at the end of 7 days. "a" indicates slight acid production. Antiserums 14, 19 and 22 were made from the corresponding cultures; M4 and M9 were secured from the H. K. Mulford Co. and reprecent Dochez types of streptococci I (S3) and III (S60), respectively. The numbers in the agglutination columns represent the highest dilution in which satisfactory and constant agglutinations were secured. The negative signs indicate no agglutination in a dilution of 1 : 50 or higher.

Absorption tests were made with all antigens, whose agglutinations were positive, by adding to a 1:5 dilution of the serum, four times its volume of antigen. The mixtures were incubated for 3 hours at 56 C. and allowed to stand over night in the icebox. The next morning they were centrifuged and the supernatant fluid used in agglutination tests with the homologous culture. If the serum showed an appreciable decline in its agglutinating powers with its homologous culture the original agglutination was considered positive.

Complement-fixation tests were made using cultures which represented the cultural groups. The antigens were heavy salt solution suspensions of broth cultures. Homologous culture and serum were used to determine the amboceptor dosage. The same quantity of antigen was used with all cultures after the proper dosage had been determined in homologous culture and serum. Complement fixation occurred indiscriminately; there was no relation between the power of fixing complement and the cultural groups. This agrees with the results of Kinsella and Swift,[4] Howell and Hitchens,[5] and others.

Bactericidal tests were carried out with the technic of Havens.[6] No relation exists between the bactericidal powers and the cultural groups, except that green serum has no effect on the hemolytic group and vice versa.

SUMMARY OF TABLE 2

The growth in broth is not recorded because of the variations in growth and morphology in the same culture at various times.

None of the organisms resembling pneumococci were bile-soluble.

Green types, as compared with hemolytic types in source, are apparently less pathogenic and in many cases purely saphrophytic in nature.

The reaction in milk seems the most distinctive single reaction we have. Green 1.1 cultures without a single exception produce acid, coagulation and reduction, while the hemolytic 1.1 cultures agree, with one exception, in their inability to coagulate and reduce milk. This one exception is from a questionable pathogenic source. On this basis we may assume that the more pathogenic have less power to affect milk than the saphrophytic. We have a specific example of this in comparing hemolytic 1.5 cultures. Those from milk are powerful in their action on milk while the one from a pathogenic source produces a moderate amount of acid in comparison with acid and coagulation in the others.

The green 1.2 cultures which the serologic reactions show to be closley related to the 1.1 group act much in the same way on milk. This group, which has its main distinction in the ability to ferment raffinose, is otherwise morphologically and culturally like the green 1.1 group.

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[4] Jour. Exper. Med., 1917, 15, p. 877.

[5] Jour. Infect. Dis., 1918, 12, p. 230.

[6] Ibid., 1919, 15, p. 315.

Serologically there seems to be no great difference between the green 1.1 and 1.2 groups. Green 1.2 serum agglutinates as many green 1.1 cultures as does a 1.1 serum, but one of the green 1.2 cultures is agglutinated by a 1.1 serum and that in a comparatively low dilution. Not all of either group are agglutinated by their culturally homologous serum. Therefore, it would seem that raffinose fermentation is not a distinctive reaction, although an antiserum from a raffinose fermenting strain has a wider range of agglutination than antiserum from a strain not fermenting raffinose.

Should Brown's sugar grouping be proved of value, the use of some system of nomenclature in connection with this chart would be advisable. The addition of the proper Roman ordinal—to represent the second half of the number—to the name already assigned which represents the first half of the number would orient the name to the number without the addition of entirely new names. It is in accordance with such a system that we suggest the name—S. secundomitis for the green 1.2 group. We have not included with the suggested names, the writers who have previously reported these organisms, as their names are listed in the chart. Nor are the morphologic characteristics included as they vary within the limits of streptococcal growth both as to individual size and length of chain.

Green 1.3 and 1.4 organisms which ferment inulin have but little power to affect milk. They produce no coagulation, and some of them are barely able to produce a trace of acidity; few produce a strong acid reaction. This inability to react on milk gives us another method of differentiating questionable streptococci from pneumococci.

These organisms are also slight acid producers in other sugar mediums. With Andrade's indicator the end-point is a faint pink. This may account for the slight reaction in milk. Otherwise, culturally, they are like S. mitis. Serologically, these two groups are distinct and separate from green 1.1 and 1.2. There have been no agglutinations in any one of them by serums from the 1.1 and 1.2 groups. Suggested names are: S. tertiomitis and S. quartomitis.

The next group, green 2.2, has its main distinction from the preceding organisms in its inability to ferment salicin. Here, again, we have two organisms from a possible pathogenic source with but little action on milk. The third from an apparently saphrophytic source is vigorous in its action on milk. The same saphrophytic organism is agglutinated by a green 1.2 serum. The best explanation for this is that the raffinose fermenters are closely related serologically. The agglutination of this nonfermenter of salicin by a salicin fermenting antiserum discards salicin from an important place in the cultural grouping. Culturally these organisms present no other distinctive reactions. S. secundosalivarius is the suggested name.

S. faecalis, green 5.1 ferments mannite and has little action on milk. These organisms attack the sugars only slightly and are not agglutinated by our serums. The green 5.3 group has the same general characteristics as green 5.1 but in addition ferments inulin. They are like the other inulin fermenters in their action on milk. S. tertiofaecalis is the suggested name.

Culture 50 represents the green 5.4 group. Morphologically it is a diplococcus, never appearing in chains. No capsule, however, can be demonstrated nor is it bile-soluble. Its reaction in milk is like that of the pneumococcus, and in addition it ferments inulin. None of our serums agglutinate it, although both green and hemolytic serums are capable of fixing complement in its presence. S. quartofaecalis is the suggested name.

In the entire hemolytic group of organisms we find 4 of them capable of producing acid, coagulation and reduction in milk. Three of these are from milk sources and 2 are typical of the S. lacticus group, while the third has the additional property of fermenting mannite. Only one hemolytic organism from a source which is likely to be pathogenic is capable of causing coagulation in milk. The agglutination positives are so few as to prevent any sweeping conclusions, but they point to the fact that the sugar fermentations are not an indication of the serologic relations.

Organism M11 ferments mannite in addition to lactose and salicin and in considering that its source is milk we may assume that it is but a variation of the S. lacticus group. S. quilacticus is the suggested name.

A summary of all the agglutination reactions places the green and hemolytic organisms in two distinct serologic groups. In no case has there been any cross agglutination between the green cultures and hemolytic serums, and the converse is equally true. The number of positive results recorded in the green group, and the way in which they arrange themselves indicates that the fundamental differences in addition to the blood reactions are those of inulin and mannite fermentation.

The pathogenesis of some of these cultures has been tested directly. They were picked at random for the test but included most of the cultural groups. None of the green cultures was able to produce death in long or short periods of time and return the organism in pure culture. In the hemolytic group 3. cultures out of five killed guinea-pigs in 24 hours, the organism being recovered in pure culture. In these tests guinea-pigs were injected intraperitoneally with 2 c c of a 24-hour broth culture.

DISCUSSION

Brown [1] states that in his studies the types previously outlined have their various characteristics in blood agar fixed and unchanging; that is, on standard culture mediums, hemolytic types do not revert to green or green to hemolytic. Havens,[6] on the contrary, states that streptococci grown on culture mediums for a few weeks after isolation lose their hemolytic powers in blood broth and become avirulent. This differs with the results in this study. Streptococci kept on blood agar for over a year now produce zones of hemolysis from 6-8 mm. in diameter in 24 hours. The same cultures when planted in blood broth still have power to cause diffusion of hemoglobin. This is not true of all cultures isolated, but, as was previously stated, these cultures were classified as hemolytic from surface platings and subsequently found not to be of the hemolytic type.

Surface plating seems responsible for a great deal of this confusion. A green organism planted on the surface of a blood-agar plate elaborates its first growth with the production of the green zone. Its secondary zone of hemolysis starts forming as in the deep colonies. The colony spreads, in its growth covering the green zone, leaving the colony in an apparent zone of hemolysis. Brown states that the secondary zone forms in the icebox or at temperatures lower that that of the incubator. This is not always true. Plates have been taken directly from the incubator to the microscope and in many cases the secondary ring is well formed. If observed within from 2-3 minutes after leaving the incubator, it seems hardly possible that the secondary ring of hemolysis could form because of the slight change in temperature in so short a time.

On this basis an investigator, using the surface method, might easily fish a green type on observing the secondary zone of hemolysis, supposing it to be a hemolytic colony. He plants it in a blood agar medium as a stock culture. In most cases that green type will color the entire tube green in a few days. Further platings, after this green has been noted, will in all likelihood class the organism as a green type. The investigator then having two results at variance with each other might be led to assume that a hemolytic organism has become a green type.

With the foregoing in mind we are adding at this point a rather detailed history of culture 19. This culture, having been roughly determined as hemolytic 1.1, was selected to immunize a pair of rabbits. Because of the necessity of having a strain of undoubted purity for the source of the antiserum, the checking which follows was done with great care and detail. On its second plating, a typical hemolytic colony grew in the depths, but on the surface there appeared a wrinkled, 2-3 mm. colony, surrounded by a greenish zone which looked neither green nor hemolytic hemolysis, but more like a green pigmentation independent of the blood itself. There was no noticeable formation of the secondary ring of hemolysis which surrounds the green formation in typical green hemolysis. This was proved a variation by fishing and finding the sugar reactions to be 1.1, replating it and finding both the new-formed wrinkled colonies and deep colonies—which were again typical—and confirming on the sugars as 1.1. On one of the secondary platings, the blood was contaminated by a green type—mentioned under Blood in the section on media—which further complicated the determination. Not all hemolytic cultures produce this type of surface growth, nor does every surface colony of this culture produce this reaction.

This type of growth corresponds closely with S. metachromatos (Brown). The following is a condensed description of the organism in a letter from Dr. Brown, "The colony on defibrinated blood agar is conical in shape, the edges irregularly serrated. The colony itself becomes brownish and has a granular appearance, with the greenish pigmentation surrounding it. When the colony is lifted, it frequently comes away in its entirety and shows the agar more or less pitted. In serum bouillon, the culture settles to the bottom and is fluffy."

These results and conjectures are inserted here because throughout the literature, there are constantly recurring statements that streptococci of various types lose or gain some of the powers of hemolysis, when grown on culture mediums for long periods of time. Our results disagree with such conclusions.

SUMMARY

Green and hemolytic types of streptococci are distinct culturally and serologically, the last measured by the agglutination reactions.

The sugar reactions are not indicators of serologic groupings, but possibly inulin fermentation and the reaction in milk may be used as of value in group determination in conjunction with the blood type.